MOLECULAR INTERACTIONS OF THE HANTAAN VIRUS NUCLEOCAPSID PROTEIN

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ABSTRACT

Title of Thesis: Molecular Interactions of the Hantaan Virus Nucleocapsid Protein

Darrell E. Griffin, III, Master of Science, 1991

Thesis directed by: John Hay, Ph.D., Professor and Vice-Chairman, Department of Microbiology

Approaches to the determination of the molecular interactions of Hantaan virus nucleocapsid protein (N) are proposed. In its role as the nucleocapsid protein, N interacts with viral RNA; however, its specificity for single-stranded or double-stranded nucleic acid and/or nucleotide sequence is unknown. The N protein is also thought to self-associate, based on previous electron microscopy and immunofluorescence assays. The specific nature of these N-N protein interactions is also uncharacterized. In addition, the extent of interactions of N with HTV glycoproteins or any other HTV proteins remains to be worked out. The interactions with protein and nucleic acid of the nucleocapsid and capsid proteins from various RNA and DNA viruses are discussed. Finally, potential experimental approaches to determine the molecular

interactions of HTV N with itself, with other proteins, with glycoproteins and with nucleic acid are described.

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by

Darrell E. Griffin, III

Thesis submitted to the Faculty of the Department of Microbiology Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Master of Science 1991

TO BETTY
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PURPOSE

The general purpose of this paper is to discuss the nucleocapsid (N) protein of Hantaan virus (HTV) in the context of what is known about its structure, expression, and function. This discussion will also include what is known about the molecular interactions of viral nucleocapsid proteins, in general, with proteins, nucleic acids, and glycoproteins. An experimental approach to determining the molecular interaction(s) of HTV N protein with proteins, nucleic acids, and glycoproteins will then be proposed.

INTRODUCTION

Bunyaviridae

More than 300 viruses isolated from all over the world are classified as belonging to the Bunyaviridae family (Karabatso, 1985). They have both spherical and enveloped virions (90-100 nm in diameter) and a single-stranded RNA genome consisting of three noncovalently closed, circular RNA species, designated small, medium, and large (S, M, and L). On the basis of serological relationships, the majority of the viruses of the Bunyaviridae have been classified into five genera: Bunyavirus, Hantavirus, Nairovirus, Phlebovirus, and Uukuvirus (Bishop, 1990). Although most of the viruses have come from hematophagous arthropods and/or vertebrates, recent analyses indicate that the thrip-transmitted plant virus, tomato spotted wilt virus, and rice stripe virus should be considered as members of the family with the proposed genus name, Phytophlebovirus (Milne and Francki, 1984; de Haan et al., 1989, 1990; Kakutani et al., 1990.)

The genome RNA of members of the Bunyaviridae range in size from 3-8 x 10^5 Da (S RNA), to 1.1-2 x 10^6 Da (M RNA),

to 2.4->4 x 10^6 Da (L RNA) (Bishop, 1990). One reason that may account for the variation in these S RNA sizes is that different viruses employ different coding strategies in this portion of their genomes (Bishop, 1990). Bunyaviruses, for example, code for two proteins (N and NS_s) that are read from overlapping reading frames in the single S viral-complementary mRNA species (Fuller et al., 1983). Hantaviruses and Nairoviruses apparently code only for an N protein in their viral-complementary sequences; no NS_s-type protein has yet been identified (Schmaljohn et al., 1986; Ward et al., 1990). Phleboviruses and Uukuviruses code for an N protein in a viral-complementary, subgenomic mRNA species corresponding to the 3′ half of the viral S RNA and an NS_s protein in a viral-sense, subgenomic mRNA corresponding to the 5′ half of the viral S RNA (Ihara et al., 1984; Simons et al., 1990). This coding arrangement is termed ambisense. Although another family of RNA viruses (the Arenaviridae) also exhibits an ambisense coding arrangement for its viral RNA species, no other similarity between the replication strategies of bunyaviruses and arenaviruses has been identified by RNA (cDNA) sequence, serological, or intracellular analyses. For viruses representing the Bunyavirus, Hantavirus, Nairovirus or Phlebovirus genera of the Bunyaviridae (or for tomato

spotted wilt virus), no nucleotide sequence similarity has been demonstrated among their S RNAs or in their encoded gene products (Bishop, 1990; Schmaljohn et al., 1986; Ward et al., 1990; Ihara et al., 1984; de Haan et al., 1990). However, recent studies have shown that the S RNA of Uukuniemi virus (Simons et al., 1990) has a similar coding strategy to that of both Punta Toro (Ihara et al., 1984) and Sicilian Sandfly fever Phleboviruses (Marriott et al., 1989). This fact, and the sequence homologies between their encoded N proteins, suggest that Uukuviruses and Phleboviruses are more closely related than had been indicated by earlier serological analyses. The S RNA of tomato spotted wilt virus also exhibits an ambisense coding strategy although no sequence relationships have been identified between its encoded gene products and those of Phleboviruses or Uukuviruses (de Haan et al., 1990).

Hantavirus

Historical

The viruses that cause Korean hemorrhagic fever (KHF), epidemic hemorrhagic fever in China, and hemorrhagic fever with renal syndrome (HFRS) in the Soviet Union are closely related and together form the genus Hantavirus (Schmaljohn

et al., 1985). KHF was first recognized by Western medicine during the Korean war. By the end of 1951, 1016 cases had been reported in soldiers of the United Nations forces with nearly 80 fatalities (Powell, 1954). A retrospective analysis of sera collected from patients with a clinical diagnosis of hemorrhagic fever has shown that the clinical diagnosis was serologically confirmed in 230 of 245 patients (LeDuc et al., 1990). KHF continues to be a problem in Korea with approximately 500-800 cases hospitalized each year (Lee, 1989). Occasional outbreaks still occur among U.S. military personnel stationed in Korea (Pon et al., 1990).

Only recently, however, through epidemiological studies, has the global nature of Hantavirus disease become clear. For example, disease analogous to HFRS has been described in most areas of the Eurasian continent. Approximately 200,000 cases of HFRS, nearly half of them in the People's Republic of China, occur annually with mortality of 2% to 10% (Song et al., 1984). In China, both the reported incidence of HFRS and the number of geographic regions affected are rapidly increasing (Chen et al., 1986).

A viral etiology for KHF was long suspected, but conclusive demonstration of such an agent did not occur unitl 1976, when antigen reactive with convalescent

patients' sera was discovered in lung tissues from the Korean striped field mice (Apodemus agrarius coreae) (Lee et al., 1978). After several passages in seronegative Apodemus, a virus, KHF strain 76-118, was isolated. Conditions for its cell culture propagation were subsequently determined (French et al., 1981). The virus was named "Hantaan" after the Hantaan River in the endemic region in northern South Korea where the first infected Apodemus was trapped (Lee, 1982). Hantaan virus (HTV) has subsequently become the prototype virus of the genus Hantavirus, in the family Bunyaviridae. Members of the genus have now been isolated from all continents with the exception of Antarctica (LeDuc et al., 1986). Whether all Hantavirus isolates are capable of causing human disease remains unknown, although it is already clear that infection with different viruses can result in a range of symptoms in humans ranging from severe to mild. Recently, epidemiological evidence of seroconversion has linked some isolates with human kidney disease of "unknown etiology" (Childs et al., 1988).

Epidemiology

Seroepidemiological investigations have shown that Hantaan virus-induced KHF is only one example of a complex

of related viruses and diseases which can be found worldwide. For example, an antigenically closely-related virus (Seoul virus), isolated from the brown rat (Rattus norvegicus), has been implicated in a milder urban form of HFRS (Lee et al., 1982). More recently, a second antigenically-related virus, isolated from the bank vole (Clethrionomys glareolus), was shown to be the causative agent of nephropathia epidemica (NE), a mild form of HFRS, which occurs mainly in Scandinavia. Evidence for the occurrence of such NE-related HFRS diseases has also been reported from most Central and Western European countries (Antoniades et al., 1985; Avsic-Zupanc et al., 1989; Brummer-Korvenkonto et al., 1980). Several NE viruses have been isolated, the type strain of which is Puumala virus (Niklasson and LeDuc, 1984; Yanagihara et al., 1984). Other recognised HTV strains include Prospect Hill virus, isolated from a meadow vole (Microtus pennsylvanicus) in Frederick, Maryland (Lee et al., 1985a; Schmaljohn et al., 1985) and Leakey virus, isolated from a common house mouse (Mus musculus) in Leakey, Texas (Baek et al., 1989). Neither Prospect Hill virus nor Leakey virus has been associated with human disease. However, serological evidence of infection with Prospect Hill virus has been found among American mammalogists (Yanagihara et al., 1984).

Serological relationships between Hantaviruses have been established using tests such as indirect immunofluorescence assays (IFA) (Lee et al., 1985b; Sugiyama et al., 1987; Saluzzo et al., 1988; Sheshberadaran et al., 1988; Zoller et al., 1989; Groen et al., 1991), plaque reduction neutralization tests (PRNT) (Lee et al., 1985b), enzyme-linked immunosorbent assays (ELISA) (Groen et al., 1991), radioimmunoprecipitation assays (RIPA) (Dantas et al., 1987; Sheshberadaran et al., 1988), immune adherence hemagglutination assays (IAHA) (Sugiyama et al., 1987), hemagglutination inhibition assays (Dantas et al., 1987), and immunoblot analyses (Zoller et al., 1989). These data indicated that there are at least five antigenically distinct serotypes based on the reactivity of viral isolates with convalescent patients' sera and with polyclonal and monoclonal antibodies directed against viral isolates. There is some cross-reactivity between the serotypes when they are tested with either convalescent patient or polyclonal sera.

Regions of the genomes of representative strains from four of the five Hantavirus serotypes have been cloned and sequenced (Schmaljohn et al., 1986; Arikawa et al., 1990; Stohwasser et al., 1990; Parrington and Kang, 1990), and relationships between the cloned strains studied through

sequence comparison. Recently the polymerase chain reaction (PCR) with subsequent nucleotide sequence analysis has been employed to detect genomic variations among different Hantavirus strains (Giebel et al., 1990). This assay has also been used in epidemiological studies, and the PCR data were consistent with those obtained by cross-neutralization, radioimmunoprecipitation, and sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Tang et al., 1990). One novel approach for detecting viral RNA which has not been reported yet with Hantaviruses is the use of riboprobes, which have been used to detect tomato spotted wilt virus in plant extracts (Huguenot et al., 1990).

The Disease

Hemorrhagic fever with renal syndrome (HFRS) is seen in diseases of varying severity which occur from the Far East to Western Europe. The most common diseases in the Far East are Korean hemorrhagic fever (KHF) and epidemic hemorrhagic fever (China), which are characterized by fever, muscular pains, hemorrhagic manifestations and proteinuria; subsequent complications of shock and renal failure often lead to death. On the other hand, the disease seen in Scandinavia, nephropathia epidemica, typifies the European

disease and is much less severe, as hemorrhagic manifestations do not occur.

KHF is an acutely prostrating fever, in which about one-third of the patients develop hemorrhagic manifestations (the remainder have influenza-like illness), with 10-15% experiencing shock leading to a mortality of 5-10%. In the typical severe case, there is a sudden onset of high fever, headache, myalgia, and severe malaise. The febrile phase lasts for 3-7 days and is followed by a hypotensive phase (of hours to several days) with petechial hemorrhages, proteinuria and thrombocytopenia; about one-third of deaths occur from hypovolemic shock during this phase. With return of blood pressure to normal or hypertensive levels, a phase of oliguria develops for 3-7 days; about one-half of the deaths occur at this time. Finally, recovery is signaled by a phase of diuresis which lasts for days to weeks; full recovery is slow and convalescence may require 6 months.

The major pathological lesions in fatal cases are disseminated hemorrhages and microscopic abnormalities in the kidneys. Occurrence and severity of kidney lesions depend on the stage of disease at which death occurred. The tubules are particularly affected and exhibit congestion, desquamation, and necrosis. In general, however, the pathogenesis of Hantavirus hemorrhagic fevers is not well

understood. Several recent studies indicate that immune complexes can be found in HFRS and that there are abnormalities in complement components, suggesting that at least some of the viral pathology may be immune-mediated.

Morphological characterization of Hantaviruses

Negative-stain and thin-section electron microscopy indicate morphologic and morphogenetic similarities between Hantaviruses and other Bunyaviridae genera (McCormick et al., 1982; White et al., 1982; Hung et al., 1985; Martin et al., 1985). However, unlike typical bunyaviruses, Hantaviruses exhibit relatively greater polymorphism, with round and elongated forms, the latter being 100 nm wide and 175 nm long (Hung et al., 1985; Martin et al., 1985). Three types of intracytoplasmic inclusion bodies, designated granular, granulofilamentous and filamentous, are another characteristic of virus-infected cells (Hung et al., 1986). These findings indicate that certain aspects of the morphogenesis of Hantaviruses differ from that of other bunyaviruses.

Electron microscopic studies with a previously unclassified virus, Thattapalyam virus, have provisionally placed this virus in the genus Hantavirus. Antigenic studies using IFA have recently confirmed the electron

microscopic data (Zeller et al., 1989).

Jennings (1987) cloned the HTV N protein coding sequences into vaccinia virus and followed the expression of N protein in infected CV-1 cells by IFA. At 10 hours post infection, the pattern of fluorescence was granular, and diffusely scattered throughtout the cytoplasm. By 24 hours, however, large rod-shaped fluorescent structures had formed; at 48 hours post infection, long strands of fluorescent material had formed and were located close to the nucleus. Jennings (1987) proposed that the long strands of fluorescence around the nucleus indicate an association with the endoplasmic reticulum and/or a self-aggregation of the nucleocapsid protein in an end-to-end fashion; however, no evidence presented. Alternatively, the self-aggregation could be in a side-to-side configuration.

Molecular characterization of Hantaviruses

Hantaviruses have a single-stranded, negative-sense RNA genome (Schmaljohn and Dalrymple, 1983). The large (L; 6.5 kb), the medium (M; 3.6 kb), and the small (S; 1.7 kb) genome segments are enclosed in three separate nucleocapsid structures which are surrounded by a lipid envelope containing two virus-specific glycoproteins designated G1 and G2 (Schmaljohn et al., 1986; Schmaljohn et al., 1987b;

Schmaljohn, 1990). The L segment is presumed to encode the virion-associated transcriptase (Elliot et al., 1984), while it has been demonstrated that M encodes the two glycoproteins, G1 and G2 (Schmaljohn et al., 1987a), and S encodes the nucleocapsid protein (Schmaljohn et al., 1986a).

Studies of HTV M and S RNA segment expression using vaccinia virus recombinants revealed that the specificity of virus neutralization appears to reside in the glycoproteins (Pensiero, et al., 1988).

Comparison of Hantavirus S RNA Segments

The Hantavirus S genome segments range in length from 1675 to 1785 nucleosides (Table 1) (Schmaljohn et al, 1986; Arikawa et al., 1990; Parrington and Kang, 1990; Stohwasser et al., 1991). In pair-way comparisons, the nucleotide identity of the sequences range from 58% to 71% (Table 2). The size differences reflect variations largely in non-coding regions of the RNAs, and may either be unimportant or, perhaps, imply differential control processes in transcription or translation of the RNAs.

Comparison of Hantavirus N Proteins

The N proteins of four viruses, which represent four of the five serotypes of Hantavirus, show a narrow range in

Table 1. Nucleotide sequences of Hantavirus S segments and amino lengths of the encoded N proteins

	S RNA Length (Nucleotides)	N Protein Length (Amino Acids)
Hantaan	1696	429
Sapporo rat	1769	429
Nephropathia epidemica	1785	433
Prospect Hill	1675	433

length (429 to 433 amino acids) (Table 2). The sequence similarity ranged from 77 to 90% and the sequence identity ranged from 62 to 82% (Table 3). Clearly, the amino acid sequences are more related than the nucleotide sequences, reflecting the need for sequence conservation in Hantavirus N proteins. The two pairs of sequence that are the most similar (HTV and SRV; NEV and PHV) also have been shown to be the most related based on serological data (Lee et al., 1985b). Comparison of these two pairs of sequences revealed that both were colinear, with no gaps in the two compared sequences, indicating that the pairs were closely related to each other. An important next step in this analysis will be to define stretches of similar or clearly different amino acid character inside the various N proteins, as a means of approaching the problem of discovering important structural features in N.

Comparison of Bunyaviridae S RNA Segments

The coding sequences of the S segment of at least one representative virus from each of the five genera of Bunyaviridae and from the proposed genus Phytophlebovirus are known (Table 4). The length of the Bunyavirus S segments range from 850 to 984 nucleotides, which is about 40% smaller than the S segments of the other genera. The S

Table 2. Comparison of the nucleotide sequences of the *Hantavirus* S genome segments

	Sapporo rat	Nephropathia epidemica	Prospect Hill
	Identical (%)	Identical (%)	Identical (%)
Hantaan	71	58	62
Sapporo rat	-	60	60
Nephropathia epidemica	-	-	66

Table 3. Comparison of the amino acid sequences of the Hantavirus N proteins

	Sapporo rat			Nephropathia epidemica			Prospect Hill		
	Similar (%)	Identical (%)	Number of gaps	Similar (%)	Identical (%)	Number of gaps	Similar (%)	Identical (%)	Number of gaps
Hantaan	90	82	0	78	63	3	77	62	1
Sapporo rat	-	-	-	77	62	1	77	63	1
Nephropathia epidemica	-	-	-	-	-	-	88	80	0

Table 4. Nucleotide sequences of Bunyaviridae S genome segments

Genus	Serogroup	Virus	Length (Nucleotides)	A+U (%)	Reference
Bunyavirus	Bunyamwera	Bunyamwera	961	58.2	Elliott, 1989
		Germiston	980	57.6	Gerbaud et al.,1987
		Maguari	945	59.9	Elliott and McGregor, 1989
	California	La Crosse	984	58.8	Akashi and Bishop, 1984
		Snowshoe hare	982	56.3	Bishop et al., 1982
	Simbu	Aino	850	55.9	Akashi et al., 1984
Hantavirus	Hantaan	Hantaan 76-118	1696	57.1	Schmaljohn et al., 1986
	Seoul	Sapporo rat SR-11	1769	58.1	Arikawa et al., 1990
	Puumula	Nephropathia epidemica Hällnäs B1	1785	56.9	Stohwasser et al., 1990
	Prospect Hill	Prospect Hill-1	1675	57.4	Parrington and Kang, 1990
Phlebovirus	Sandfly fever	Toscana	1869	52.9	Giorgi et al., 1991
	Rift Valley fever	Rift Valley fever	1690	50.9	Giorgi et al., 1991
	Punta Toro	Punta Toro	1904	60.4	Ihara et al., 1984
	Ungrouped	Sandfly fever Sicilian	1746	53.2	Marriott et al., 1989
Nairovirus	Nairobi sheep disease	Dugbe	1712	56.5	Ward et al., 1990
Uukuvirus	Uukuniemi	Uukuniemi	1720	50.0	Simons et al., 1990
Phytophlebovirus		Tomato spotted wilt	2916	64.5	de Haan et al., 1990

segments of the phleboviruses range from 1690 to 1904 nucleotides. The lengths of Dugbe virus (Nairovirus) and Uukuniemi virus (Uukuvirus) are about the same - 1712 and 1720 nucleotides, respectively. The S segment of the tomato spotted wilt virus is considerably larger (2916 nucleotides) than S segments of the other Bunyaviridae. Like the Hantavirus members, each of the other genera's S RNAs have characteristic complementary 5′ and 3′ terminal sequences. In this set of analyses, we are presumably seeing length differences which are largely a reflection of the different coding strategies employed by these viruses; it is surprising, perhaps, that such basic differences exist among viruses of the one family.

Comparison of Bunyaviridae N Proteins

The coding strategy of the genomic S RNA species and the predicted amino acid composition of the encoded proteins have been determined (Table 5). Viruses of the Bunyavirus genus have S segments that encode a N protein and a non-structural protein, NS_s, in overlapping reading frames. The sizes of the bunyaviral N and NS_s proteins range from 233 to 235 amino acids and from 75 to 109 amino acids, respectively. Like the Hantaviruses, Dugbe virus (Nairovirus) produces only a N protein which is 442 amino

Table 5. Coding capacity of Bunyaviridae S genome segments

Genus	Serogroup	Virus	Protein	Number of amino acids	Predicted M_r (10^3 daltons)	Reference
Bunyavirus	Bunyamwera	Bunyamwera	N NS$_s$	233 101	26.7 11.0	Elliott, 1989
		Germiston	N NS$_s$	233 109	26.6 11.8	Gerbaud et al.,1987
		Maguari	N NS$_s$	233 75	26.0 11.0	Elliott and McGregor, 1989
	California	La Crosse	N NS$_s$	235 92	26.5 10.4	Akashi and Bishop, 1983
		Snowshoe Hare	N NS$_s$	235 92	26.8 10.5	Bishop et al., 1982
	Simbu	Aino	N NS$_s$	235 91	26.2 10.8	Akashi et al., 1984
Hantavirus	Hantaan	Hantaan 76-118	N	429	48.2	Schmaljohn et al., 1986
	Seoul	Sapporo rat SR-11	N	429	48	Arikawa et al., 1990
	Puumula	Nephropathia epidemica Hällnäs B1	N	433	49	Stohwasser et al., 1990
	Prospect Hill	Prospect Hill-1	N	433	49.0	Parrington and Kang, 1990
Phlebovirus	Sandfly fever	Toscana	N NS$_s$	254 317	27.7 36.7	Giorgi et al., 1991
	Rift Valley fever	Rift Valley fever	N NS$_s$	246 266	27.4 29.9	Giorgi et al., 1991
	Punta Toro	Punta Toro	N NS$_s$	243 250	26.9 29.1	Ihara et al., 1984
	Ungrouped	Sandfly fever Sicilian	N NS$_s$	219 268	24.8 30.4	Marriott et al., 1989
Nairovirus	Nairobi sheep disease	Dugbe	N	442	49.4	Ward et al., 1990
Uukuvirus	Uukuniemi	Uukuniemi	N NS$_s$	258 273	28.5 32.0	Simons et al., 1990
Phytophlebovirus		Tomato spotted wilt	N NS$_s$	259 465	28.8 52.4	de Haan et al., 1990

acids in length. The S RNAs of the Phlebovirus members have an ambisense coding arrangement. For Punta Toro virus, the 3′ half codes in a viral-complementary sequence for the 27,000Da N protein, and the 5′ half codes in a viral-sense sequence for the 29,000Da NS_s protein (Ihara et al., 1985). The intergenic region is rich in stretches of A and U residues and can be arranged into a hairpin configuration that serves as an mRNA transcription terminator (Emery et al., 1987). Both N and NS_s mRNA species are subgenomic and are approximately half the size of S RNA (Ihara et al., 1985). The length of the phleboviral N proteins are from 219 to 254 amino acids; the length of the NS_s proteins are from 250 to 317 amino acids. The S RNA of Uukuniemi virus (Uukuvirus) also has an ambisense coding arrangement with a N protein (258 amino acids) and a NS_s protein (273 amino acids) (Simons et al., 1990). The S RNA of tomato spotted wilt virus uses an ambisense coding arrangement with a N protein (259 amino acids) and a NS_s protein (465 amino acids). The NS_s of tomato spotted wilt virus is much larger than the bunyaviral, phleboviral, and uukuviral NS_s proteins (de Haan et al., 1990).

As mentioned above, we are seeing, in this comparison, differences in strategy among the Bunyaviridae. Nevertheless, inside the coding regions for the N proteins

Nevertheless, inside the coding regions for the N proteins (which all members possess) there will be similarities and differences evident from further analysis of the sequences. Analyzing these would be one of the first items in the proposed project, and the resulting data would be used to design appropriate mutants for study, among other things.

DISCUSSION

The interactions of proteins found in the nucleocapsids of a wide range of viruses have been characterized in a variety of ways. Examples of their molecular interactions with themselves, other proteins, RNAs, DNAs, and glycoproteins will be discussed below.

Protein-Protein Interactions of Nucleocapsid Proteins

The major capsid protein, VP1, of the small DNA virus, polyomavirus, has been cloned into and expressed from Escherichia coli, and the recombinant protein has been purified to near-homogeneity (Leavitt et al., 1985). This recombinant VP1 protein can be prepared from E. coli as pentamers which resemble virion capsomeres, and these pentamers self-assemble into capsid-like aggregates in vitro. These "capsids" are readily isolated by velocity sedimentation analysis in sucrose gradients and can be visualized by electron microscopy (Salunke et al., 1986).

Proteins from Sendai (paramyxovirus) virus particles and from Sendai-infected cells have been analyzed in a protein-blotting protein-overlay assay for their interaction with in vitro-synthesized, [^{35}S] methionine-labeled viral

nucleocapsid protein (NP), RNA polymerase protein (P), and matrix protein (M). After separation by SDS-PAGE, transfer onto polyvinylidene difluoride membranes and renaturation, the immobilized proteins were found to interact specifically with certain radiolabeled proteins. NP proteins from virus particles and from infected cells interacted with [^{35}S]-P protein equally well. Conversely, P proteins from virus particles and from infected cells interact with [^{35}S]-NP protein. The M protein appeared not to be interactive in this system. To determine the domains on the NP protein required for binding to immobilized P protein, a series of truncated and internally deleted ^{35}S-NP proteins was constructed. Interestingly, the only deletion which did not affect binding involved residues 426 through 497. For example, the carboxyl-terminal 27 residues (positions 498 to 524) contribute significantly to the binding affinity, and removal of 20 residues (positions 225 to 244) in the hydrophobic middle part of NP protein completely abolishes its binding to P protein (Homann et al., 1991). These results contrast with those obtained with vesicular stomatitis virus (VSV; a rhabdovirus), where the phosphoprotein NS (analogous to Sendai P protein) contains a single carboxyl-terminal domain responsible for interaction with the nucleocapsid protein (N), and an adjacent domain

not involved in nucleocapsid recognition, but required for RNA synthesis *in vitro* (Gill *et al.*, 1986; Emerson and Schubert, 1987). Sendai virus P protein similarly requires residues near the carboxyl-terminus for attachment to nucleocapsids, but a second P protein domain is also required for this function (Ryan and Pornter, 1990). Thus, it is clear that the mechanism behind specific binding of proteins to themselves or to other proteins is a complex process whose basic features may vary greatly from virus to virus.

The N (nucleocapsid) protein of VSV, prepared in soluble form from intracellular nucleocapsids of cells infected with VSV, was found to self-assemble, as we saw above with the major capsid protein of polyoma virus. Clear, concentrated solutions of N (1-2 mg/ml) became increasingly opalescent on warming; the same opalescence appeared at 0°C when the pH was lowered to 6. At this point, numerous doughnut-like structures reminiscent of rhabdovirus capsomeres were clearly visible in the electron microscope (Blumberg *et al.*, 1983). This innate ability of viral structural proteins to interact with themselves and with other proteins is an essential feature of the ability of virus particles to be assembled from their component parts in the "production line" of virus multiplication. As noted

earlier, the HTV nucleocapsid protein is capable of "self-assembly" (aggregation) if it is synthesized in sufficient quantity in the infected cell; this property, we would predict, represents an important functional determinant of the protein.

RNA-Protein Interactions of Nucleocapsid Proteins

There are several examples of nucleic acid-protein binding specificity in the encapsidation of viral nucleic acids. The best-defined system of virus assembly is that of tobacco mosaic virus (TMV), an RNA plant virus whose nucleocapsid (virion) exhibits helical symmetry. TMV virion assembly is initiated by specific interaction between the coat protein disk (one turn of the helix containing side-by-side multimers of the nucleocapsid protein) and an internal sequence in the RNA genome called the origin-of-assembly sequence (OAS) (Blommer and Butler, 1986). This RNA sequence contains a putative stem-loop structure, believed to be the target for disk binding in assembly initiation, which has the characteristic sequence AAGAAGUCG exposed as a single strand on its apex. The 75-base RNA sequence encompassing the stem-loop is sufficient to direct the encapsidation by TMV coat protein disks of a heterologous RNA fragment (Turner et al, 1988). The

temporal order in which the addition of further nucleocapsid protein to the RNA strand (leading to complete encapsidation of the genome) takes place has been studied using RNA transcripts with the TMV OAS embedded at different positions in heterologous RNA. This facilitated the analysis of packaging in both directions during in vitro reconstruction. The packaging in the 3′ direction was shown to be slower and does not appear to commence until 5′ rod formation (packaging) is complete (Gaddipati and Siegel, 1990).

In Sindbis virus (a togavirus), sequences in the genomic RNA have also been identified that interact specifically with the capsid protein; this can be seen in in vitro binding assays using purified capsid protein and RNA transcripts. The sequences of the genome required for binding to the capsid protein are located near the 5′ terminus of the RNA, and were not essential for viral RNA replication. However, RNAs from defective interfering particles that lacked these sequences were not detected in passaged virus (Weiss et al., 1989).

In addition, a soluble form of VSV (rhabdovirus) N protein was found, in the presence of various RNAs, to assemble into RNAase-resistant structures with the buoyant density of viral nucleocapsids. The process was selective for VSV leader RNAs or other VSV transcripts.

Interestingly, however, the basis for this selective encapsidation was not the relative size of the viral transcripts nor the presence or absence of a 5′ cap structure, but was solely sequence-dependent. Partial-assembly experiments demonstrated that leader RNA assembly started within the first 14 nucleotides at the 5′ end. Subsequent examination of known leader RNA sequences suggested that the element responsible for selective assembly by VSV N protein is a five-times-repeated adenosine residue at every third position, starting from the 5′ end of the leader RNA sequence (Blumberg et al., 1983).

La Crosse virus is a member of the California encephalitis serogroup of the Bunyaviridae. The viral genome (and antigenomes) are found as circular, helical nucleocapsids, assembled with the nucleocapsid protein (N), in a structure which is sufficiently stable to survive CsCl density gradient centrifugation. La Crosse nucleocapsid (N) protein recognizes a specific binding site on its own RNAs, both genomic and antigenomic (mRNA) and, once the initial interactions have taken place, further N binding would presumably become cooperative due to N-N interactions, and assembly of genomic and "antigenome" nucleocapsids would ensue. Because the N binding site is highly specific, this interaction is limited essentially to viral RNAs, and both

genomic and "antigenomic" (message) RNAs possess the appropriate sequence at their 5′ termini. The sequence is the conserved region characteristic for each bunyavirus genus, which we discussed earlier. This process appears to be used by the virus in the earlier phases of infection as a means of controlling protein expression, while authentic nucleocapsids may be formed at later times. For example, within 24 to 48 h of La Crosse infection of mosquito cells, more than 75% of the S mRNA is found to band in CsCl density gradients at the position of antigenome (or genome) nucleocapsids. The encapsidation of the S mRNA correlates with the repression of N protein (encoded in S) synthesis _in vivo_; it can be shown that encapsidated S mRNA cannot be translated _in vitro_. Thus, the authors suggest, a pool of unassembled N protein in infected cells increases to the point at which it begins to interact with its own mRNA. This leads to the "coating" of the entire mRNA, except, interestingly enough, for the nontemplated primer required for RNA replication. Since a continuous supply of unassembled N protein appears to be required for genome synthesis and is therefore "mopped up" in this process, the infection may become self-limiting through the unavailability of N protein for assembly of authentic (genomic) nucleocapsids (Hacker _et al._, 1989). Indeed, La

Crosse virus fails to complete genomic nucleocapsid production in mosquito cells, probably for the above reasons.

RNA-binding proteins of coronavirus mouse hepatitis virus (MHV) strain A59 were identified using an RNA overlay-protein blot assay (ROPBA). The major RNA-binding protein in the virion and in infected cells is the 50KDa phosphorylated nucleocapsid protein (N). A previously-unreported 140KDa virus structural protein was identified as a minor RNA-binding protein both in virions and infected cells. Subsequent work showed that the 140KDa protein was antigenically related to N, and upon reduction, yielded only 50KDa N protein. Thus, the 140KDa species is probably a trimer of N subunits linked by intermolecular disulfide bonds. The RNA binding ability of MHV N protein was not nucleotide sequence-specific, in the assay used. Single-stranded RNA of MHV, VSV, or of cellular origin, a DNA probe of the MHV leader sequence and double-stranded bovine rotavirus RNA were all able to bind to N. Binding of MHV RNA was optimal between pH 7 and 8, and the RNA could be eluted in 0.1 M NaCl (Robbins et al., 1986). In an in vitro assay system, domains I and III of the MHV N protein were found to be dispensable for binding to RNA, suggesting that the RNA-binding characteristic of N resides in domain II

((Masters et al., manuscript in preparation) in Parker and Masters, 1990). The leader RNA of MHV, which acts as a primer for mRNA synthesis, is also specifically bound by the N protein. It has not been determined, however, if this interaction affects translation and if this might represent a mechanism for controlling MHV gene expression, as we saw above for La Crosse virus (Stohlman et al., 1988).

The hepatitis delta virus (HDV; unclassified) antigen (HDAg) has been shown to be an internal viral structural protein which binds specifically to HDV RNA; however, HDAg does not form a classical (i.e. neither icosahedral nor helical) nucleocapsid structure. The specificity of HDAg binding has been demonstrated by two independent methods: a Northwestern blot procedure (RNA binds to protein on a blot) and an RNA mobility shift assay. The binding in both assays occurred at relatively high salt concentration, and could be competed out by homologous RNAs, but not by other RNAs. It was determined, surprisingly, that this specific binding was not the result of the presence of a specific nucleotide sequence but rather of the formation of a specific RNA conformation unique to HDV RNA. The binding domain on the HDAg protein is also specific; only the middle domain of the protein binds HDV RNA. Finally, at least some of the HDAg binds to circular HDV RNA via a bond which is resistant to

treatment with 0.1% SDS and 0.5% Nonidet P-40 (Lin et al., 1990) and therefore may well be covalent in nature.

The influenza virus (orthomyxovirus) nucleocapsid is made up of eight separate segments of single-stranded, negative-sense RNA. In the virion and in isolated ribonucleoproteins (RNPs), the RNA is in the form of a helix coated with the viral nucleoprotein (NP). While the influenza RNA polymerase protein complex is also present in small amounts in nucleocapsids, NP is the only protein present on the RNPs in high copy number (one NP per 20 nucleotides) (Compans et al., 1972). The binding of NP to RNA has recently been analyzed. NP purified from virions along with in vitro-synthesized influenza RNA were used both in filter binding and gel retardation assays. Reconstituted NP-RNA complexes were then analyzed by RNase "reverse-printing" method, using RNase V1, which revealed that reconstituted NP-RNA complexes carry RNase V1-sensitive sites as do native RNP cores; this implies that RNA-NP complexes, structurally similar to native RNP cores, are reconstituted from isolated components (Yamanaka et al., 1990).

Morgan (1991) has observed recently that all of the paramyxoviruses (Sendai virus, measles virus etc.) with the exception of respiratory syncytial virus, contain three

clusters of conserved amino acids in the central portion of the nucleoprotein (NP) molecule. Since conservation of common sequences in two genes presumably signifies functional similarity (Dover and Flavell, 1984), these regions may represent potential binding sites for the L (RNA polymerase) and P (phosphoprotein) proteins or for interaction with other NP (nucleocapsid) molecules or with genomic RNA. It has been shown that the carboxyl-terminal region of the Sendai virus NP could be removed by tryptic digestion without altering the structure of the nucleocapsid (Heggeness et al., 1981), whereas the amino half of the protein is believed to interact directly with the virion RNA (Morgan et al., 1984).

A potentially-useful affinity chromatography method for the isolation of specific RNAs and RNA-protein complexes formed in vivo or in vitro has been described by Bardwell and Wickens (1990). It exploits the highly selective binding of the coat protein of bacteriophage R17 to a short 19-base hairpin in its genomic RNA. RNA containing that hairpin binds to coat protein that has been covalently bound to a solid support. Bound RNA-protein complexes can be eluted with excess R17 recognition sites. Using purified RNA, they demonstrated that binding to immobilized coat protein is highly specific and enables one to separate an

RNA of interest from a large excess of other RNAs in a single step.

In attempts to accomplish similar ends, RNA-protein cross-links were introduced into brome mosaic virus *in situ* using the photoreactive, heterobifunctional agent *p*-azidophenylglyoxal. An improved RNA isolation procedure, without phenol extraction, was used to isolate RNA cross-linked with protein. RNA of the covalently linked complex was acid-digested and the oligonucleotides sill attached to proteins were 5′-end-labelled with ^{32}P. The complexes were digested with trypsin and the tryptic peptides were purified by reversed-phase high-performance liquid chromatography. Amino acid analyses of cross-linked tryptic peptides revealed that out of the total 188 amino acids of the brome mosaic virus coat protein only the 80 N-terminal amino acids are involved in the interaction with viral RNA (Sgro *et al.*, 1986).

In retroviruses, the nucleocapsid protein (NC) is a small, highly basic, protein derived from the *gag* gene. NC is complexed with two identical single-stranded RNA molecules (Dickson *et al.*, 1982) in the retrovirus virions. The molar ratio of NC to RNA in viral particles suggests that the genomic RNA molecule is essentially coated with NC (Karpel *et al.*, 1987), while cross-linking studies suggest

that NC may bind more tightly to certain regions of the RNA genome than to others (Meric et al., 1984). In vitro, NC displays nonspecific, single-stranded nucleic acid binding activity (Leis and Jentoft, 1983) and one molecule can cover 4-6 nucleotides (Karpel et al., 1987). A cysteine-histidine region, usually composed of 14 amino acids and reminiscent of "zinc fingers" in transcription factors, is the only highly conserved sequence element among the retroviral NC proteins (Katz and Jentoft, 1989), and may therefore be important in nucleocapsid formation.

As can be seen then, from the above discussion, there is ample evidence for specific interactions between viral proteins and their genomic (and sometimes other) RNAs. In most cases, there seems to be recognition by specific elements in the protein structure with short, defined stretches of nucleotide sequence, often at or near the ends of the molecule. Nevertheless, as outlined above, certain viruses have adopted other means of ensuring specific nucleic acid-protein interaction. The key factor underlying all of these processes, however, is that they have to take place in order to allow proper growth and development of the virus.

DNA-Protein Interactions of Nucleocapsid Proteins

In experiments to investigate the formation of possible intermediates in polyoma virus virion formation, subviral structures were seen in infected cells, suggesting that viral capsid proteins (VP1) are incrementally added to a 75S minichromosome to form the final 240S virion (Garcea and Benjamin, 1983). More recently, the interaction of recombinant VP1 protein with DNA has been studied using a nitrocellulose filter transfer assay. With VP1 proteins deleted at the carboxyl and amino termini, a region of the protein affecting DNA binding was identified within the first seven amino acids of the VP1 amino terminus. The DNA binding constant of the recombinant VP1 was high (10^{-11} M); however, the DNA binding is non-sequence-specific since the plasmid DNA was bound as well as the polyomavirus DNA (Moreland et al., 1991). While papovaviruses are known to package cellular sequences, this happens on a sufficiently small scale to suggest that indeed there is a mechanism which confers specificity on the encapsidation process; clearly it remains to be discovered.

Herpesviruses appear to package their DNA genomes into preformed icosahedral capsids and the genome does not seem to be intimately associated with any protein in the virion. According to Deiss et al. (1986), viral protein(s) (as yet

unidentified) with specific affinity for "a" sequences in the joint region between monomers of the viral genome (replicated as concatemers) attach to DNA, which is then taken up into empty capsids until a "head-full" is engulfed. At this point, a nuclease activity cuts the DNA, releasing the remains of the concatemer to interact with other capsids. One of the proteins thought to be involved in this process is the herpes simplex virus type 2 38kDa DNA-binding protein (Burdett and Docherty, 1987), which has been shown to be a component of the nucleocapsid, and appears to be intimately associated with the nuclear matrix throughout the infectious cycle (Burdett et al., 1990).

Nucleocapsid Interactions with Glycoproteins

The specific recognition of viral glycoproteins by the nucleocapsid protein(s) helps to ensure proper envelopment and virion maturation in a number of viruses. One classic example is the alphavirus, Semliki Forest virus. The specific interaction between the spike glycoprotein and the nucleocapsid was investigated by treating isolated virions with the non-ionic detergent, ß-D-octylglucoside, at neutral pH and low ionic strength. The interaction between the nucleocapsid and glycoprotein was sensitive to this treatment, as well as to elevated pH and ionic strength

(Helenius and Kartenbeck, 1980), suggesting that relatively weak non-covalent forces were involved. Further studies using idiotypic antibodies have provided evidence for a specific interaction between the cytoplasmic tail of the E2 glycoprotein and the nucleocapsid (Weiss et al., 1989).

The structural organization of the arenavirus, lymphocytic choriomeningitis virus (LCMV), has also been examined with detergent treatment. Triton X-114 phase separation and nearest neighbor analysis were used to define the protein-protein interactions in the virion. This treatment, along with membrane permeable and membrane impermeable crosslinking reagents, established the structural organization of the virion. Protein complexes composed of the glycoprotein GP-2, which is an integral membrane protein, and nucleocapsid protein (NP) were observed after treatment with a membrane permeable crosslinker (dimethyl suberimidate-2HCl) but not after treatment with the membrane impermeable crosslinker (3,3'-dithiobis(sulfo-succinimidylpropionate)), localizing the site of the GP-2:NP interaction to the interior of the virion. The interaction of GP-2 with NP may be important in directing the maturation and budding of LCM virions (Burns and Buchmeier, 1991).

In an analogous fashion, evidence for interaction of

the nucleocapsid of MHV strain A59 (coronavirus) with the membrane glycoprotein E1 was found after solubilization of viral membranes with Nonidet P-40 at 4°C and incubation at 37°C. The complex between E1 and the viral nucleocapsid was caused by a temperature-dependent conformational change in E1 which resulted in aggregation of E1 and interaction with the viral RNA in the nucleocapsid. The complex consisted of the nucleocapsid protein (N), RNA, and E1; however, E1 did not interact directly with the N protein (Sturman et al., 1980).

Proposed Approaches to Study Molecular Interactions of HTV N

In considering possible approaches to define the molecular interactions of HTV N protein, we are theoretically in a position to use the "natural" virus-cell system (HTV-infected Vero or human endothelial cells), with appropriate specific antiviral antibodies, as well as a battery of genetic constructs which express both the N protein in apparently authentic form, as well as viral RNAs of genomic or "antigenomic" sense. Thus, these potentially vital interactions can be studied under both in vitro and in vivo conditions using HTV itself or recombinant nucleocapsid protein. However, taking a practical view of the problem, we will confine our discussion of the proposed approaches to

the use of recombinant N protein, since HTV is a Class III biological agent, has low virus yield, and grows slowly. In addition, as noted above, it has been possible in a variety of other systems to define quite adequately the kinds of interactions we would be interested in, using wholly in vitro systems. Once interactions have been determined with the recombinant expressed N protein, specific points might then be addressed using the HTV-infected cell system.

The HTV S segment has been cloned into various bacterial expression systems (Jennings, 1987; Schmaljohn et al., 1986), into a vaccinia virus vector system (Jennings, 1987; Pensiero et al., 1988), and into baculovirus (Schmaljohn et al., 1988b). Mouse and rabbit polyclonal antibodies against HTV-infected cells are available from USAMRIID, while panels of mouse monoclonal antibodies against the N protein from the HTV prototype strain 76-118, Seoul virus strain R22, and Puumala virus are available from the Centers for Disease Control (Ruo, S., pers. comm.). Rabbit antibodies to the carboxyl terminal eleven amino acids of N protein (Jennings, 1987) and to vaccinia-expressed N protein (Pensiero, M., pers. comm.) are also available.

Since the sequences of the S segments from four strains of Hantavirus are known, sequence comparisons can be made by

computer analysis to determine the extent of homology on both the RNA and protein levels. Analysis of the sequence homologies on the RNA level may give insight to the regions of the RNA that may be important in nucleocapsid formation. The sequences of the L genome segment of HTV and NEV (Stohwasser et al., 1991) and of the M genome segment of HTV, HoJo strain (Schmaljohn et al., 1988a), Lee strain (Schmaljohn et al., 1988a), NEV (Giebel et al., 1989), Sapporo rat (Arikawa et al., 1990), Biken-1 strain (Isegawa et al., 1990), Seoul 80-39 strain (Antic et al., 1991), and Prospect Hill (Parrington et al., 1991) are available for comparison with the S genome segments. Analysis of the deduced amino acid sequences of the Hantavirus N proteins can also be used to identify (common) regions of the N protein that may be involved in RNA-protein and protein-protein interactions.

Understanding the interaction of the HTV N protein with RNA would be important to understanding how the nucleocapsid is formed. Since HTV has three genome segments (Schmaljohn et al., 1983), there is likely to be a common encapsidation signal on each of the segments. The 3′-terminal nucleotide sequence of the three HTV genomic RNA segments have a common consensus sequence of AUCAUCAUC; sequences complementary to those at the 3′ termini have been identified on the 5′

termini (Schmaljohn et al., 1986; Schmaljohn et al., 1987b; Schmaljohn, 1990). The complementary ends have been postulated to allow formation of stable, base-paired, panhandle structures (Bishop, 1990). It would be important to determine whether the N protein interacts with either the 3′ ends, the 5′ ends, or the base-paired complementary ends. Jennings (1987) attempted to modify an in vitro assay used by Kingsbury et al. (1987) to study the encapsidation of HTV RNA using synthesized oligonucleotides corresponding to the conserved sequences of the 3′ terminus of the S segment RNA as well as a 72-mer oligonucleotide corresponding to the 36 nucleotides from each end (3′ and 5′) of the S segment. The oligonucleotides were radiolabeled, incubated with an N protein preparation, and the mixture was filtered through nitrocellulose. The presence of RNA-protein complexes retained on the filter could detected by the presence of radioactivity, since unbound RNA would not be retained. Unfortunately, Jennings was unable to purify N sufficiently to eliminate background from other RNA binding proteins in his assay.

We would propose to adopt this same approach, but ensuring that our N protein was purified using a number of potentially-useful steps to eliminate other RNA-binding proteins. These would include DEAE cellulose,

phosphocellulose and gel filtration, as well as a nucleic-acid-binding column. The N protein could be detected using Western blots during the purification scheme. An alternative, and perhaps better method of purifying N would be to use the rabbit antibody against vaccinia-expressed N, which has been absorbed with vaccinia vector alone, to purify N protein by immunoaffinity chromatography. It may be important to disassociate aggregated N protein. This could be accomplished by varying ionic conditions or by using either non-ionic or ionic detergents. The specificity of the N-RNA interaction could be then be tested using the oligonucleotides mentioned above, as well as several oligonucleotides from the middle of the N gene, vector alone, RNA from other virus families, and viral and nonviral DNA.

The assay used in previous attempts to detect N-RNA interactions was the filter-binding assay. One of its disadvantages is that the specific protein species responsible for the binding cannot be determined unless pure protein preparations are available. In other systems (see above) Northwestern blot assays have been successfully used to demonstrate the RNA interactions of, particularly, HDAg and MHV N, respectively (Robbins et al., 1986; Lin et al., 1990). We would also attempt to use this approach as an

adjunct to the filter assay. In addition, if the signal on the genomic RNA for N protein binding lies (as we suspect it might) in a short terminal oligonucleotide (see above), it should be possible to carry out "gel shift" assays which, combined with the use of appropriate antibodies to N protein, should allow definition of the specific interaction, if one exists.

If a specific interaction between N and HTV genomic RNA is found using any of the above methods, then the region of RNA responsible for the interaction could be determined by making deletions, 3'-terminal truncations or 5'-terminal truncations in the subcloned genomic RNA. If a specific encapsidation signal is found, it can be cloned into heterologous RNA to confirm its function, as well as allowing investigations of the nature of the encapsidation process.

The region(s) of N protein responsible for the RNA interaction can also be delineated by making deletions, N-terminal truncations and C-terminal truncations in the N protein-coding regions to create appropriate modifications of the protein structure. An alternative approach to determining the regions of N responsible for specific RNA binding would be to make protein fusions with the E. coli maltose binding protein (Amann and Brosius, 1985; Kellerman

and Ferenci, 1982). These fusion proteins could contain both amino- and carboxyl-terminal deletions, would be relatively simple to construct and isolate and could also be used to make a battery of useful N-specific antibodies.

The protein-protein interactions of N could be determined using the protein-blotting protein-overlay system successfully employed for Sendai virus (Homann et al., 1991). In vitro-translated Hantaan proteins and glycoproteins (N, G1 and G2) could be labeled with ^{35}S and overlaid on renatured blotted N preparations. In addition the formation of aggregates (crystals?) of N protein visible in vaccinia-N recombinant-infected cells (see earlier) could be used as an assay for N-N interactions. Clearly, we would wish to use the truncated versions of N protein described earlier, in attempts to define regions of the protein important for this self-association property.

The interaction of N and HTV glycoproteins could also be demonstrated in vaccinia-recombinant infected cells using coinfections of N-expression and G1- and/or G2-expressing vaccinia strains. These recombinants have already been constructed, and the interaction could readily be visualized in an immunofluorescence assay using monospecific or monoclonal antibodies against expressed HTV proteins that have been labelled with a different fluorochrome. Using a

confocal scanning optical microscope instead of a conventional epi-fluorescent microscope would improve the resolution (Shotton, 1989). On a molecular level the interaction of N with the glycoprotein(s) could be shown using cross-linking reagents as done with LCMV (Burns and Buchmeier, 1991). Again, it would be sensible at this stage to employ modified (mutated) constructs of the HTV proteins in vaccinia to define the precise regions of amino acid sequence, given the successful demonstration of interactions with the intact molecules.

The interest in these studies, of course, is in the fundamental nature of protein-RNA and protein-protein interactions for the infectious processes involved in HTV multiplication. Our working hypothesis is that, to allow formation of the HTV virion, first nucleocapsid protein (N) molecules must (selectively) recognize HTV genomic RNA and bind to it, then, probably cooperatively, coat the entire RNA strand through protein-protein (N-N) interactions. Next, for envelopment of the nucleocapsid(s), these must migrate to the Golgi apparatus (the site of budding of HTV) and be retained beside portions of the Golgi membrane, suitably decorated with HTV glycoproteins, to allow the final step in the formation of the HTV particle, the wrapping of the nucleocapsids in the viral envelope. We hypothesize (and

our experiments are designed to test this) that N protein may contain signals for Golgi migration and/or Golgi retention, and that the nucleocapsids (through N or the N-RNA complex) are able to interact with viral glycoproteins so that budding is able to take place at an appropriate region of the Golgi membrane.

While these molecular interactions are central to the infectious process and would form the basis for the work proposed here, we are aware that other important interactions are likely to be an integral part of the HTV cycle. For example, the process of transcription and replication of the viral genome must involve recognition of RNA sequences or structures (e.g. RNA-N complexes) by the polymerase enzyme, and this polymerase activity might itself be a complex constructed from a number of polypeptides which interact specifically. Then, potential control mechanisms to adjust levels of replication and transcription might, as we have discussed above, involve special interactions between proteins and/or RNA, or might even be dependent on particular cellular localization. Also, at the level of entry of the virus into the cell, it is likely that a specific interaction(s) are required between a viral protein (probably G1 and/or G2) and a cellular membrane (receptor) protein.

The final outcome of this work with viral maturation would be to attempt to duplicate the events in the infected cell in an *in vitro* system, so that a detailed picture of the process could be drawn up. Realistically, the aim would be to initially to set up an assay for the encapsidation of genomic RNA by N protein in the "test tube" as a first step in this investigation. This could be achieved using available reagents to make protein and RNA and would use electron microscopy and biochemical techniques to detect the products of interactions.

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